Attorney Docket: Ishii Case 15

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:Yoshikazu MATSUDA
Serial	: 09/834 290
Filed	: April 12,2001
For	: HIGH-MINERAL OYSTER EXTRACT AND A PROCESS FOR MANUFACTURING THE SAME
Art Unit	: 1761
Examiner	: CORBIN ARTHUR L

DECLARATION UNDER 37 CFR 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR ;

I, Yoshikazu MATSUDA, declare as follows:

1. I am the sole inventor of the above-identified patent application.

2. The following Experiments were carried out under my supervision to prepare a zinc rich powder (ZRP) from oyster extraction residue regarding to the present invention.

a. The dried powder of the hot water extraction residue of oyster was mixed with 20 volumes of 0.1M HCl.
b. The mixture remained at room temperature with shaking for 16 h, and was then centrifuged at 750 x g for 20 min.
c. The supernatant was neutralized to pH 7.0 by the dropwise addition of 1.0M NaOH.
d. The precipitation was collected by centrifugation at 6,000 x g for 20 min.
e. The precipitation was freezedried and used as ZRP (zinc rich powder) of oysters.

3. The chemical composition of ZRP (zinc rich powder) was showed in Table 1.

Table 1. Mineral content of ZRP (zinc rich powder) of oysters

Minerals	Content (mg/g)	
	ZRP	Raw oyster
Zinc	76.0	2.57
Sodium	62.5	
Phosphorus	46.5	
Iron	4.70	
Calcium	22.4	1.45
Potassium	4.00	
Magnesium	4.05	5.7
Copper	2.78	
Manganese	1.26	0.084

4. The following Experiments were carried out to evaluate the advantages of the bioavailability of ZRP (zinc rich powder).

a. Four-week-old male Wister rats (n=18), weighing 40 to 50g each, were housed in stainless steel wire mesh hanging cages at a temperature of 22 to 24 °C and a humidity of 60 % in a room with a controlled 12 h light and dark cycle.

b. The first group of the rats was fed with a casein-based low zinc diet, the second group was fed with the basal diet with 5 μg/g of zinc as zinc carbonate hydroxide, and the third group was fed with the basal diet with 5 μg/g of zinc as ZRP (zinc rich powder).

c. After feeding for 4 weeks, the blood, liver, muscles and tibia were isolated, washed, blotted and weighted.

d. The rat tissue was mixed with HNO_3 and heated in a boiling water bath until the insoluble components disappeared.

e. The acid digests were then diluted with deionized water, the zinc level in the diluted acid digests was determined with an atomic absorption flame emission spectrophotometer.

f. The zinc analysis was verified using standard reference materials (RM 8414, bovine muscle powder, National Institute of Standard & Technology, USA).

g. The zinc deposition in the plasma, erythrocytes, liver, muscles and tibia were summarized in Table 2.

Zinc in the tibia varied according to the zinc source, ZRP supplementation significantly increased the zinc deposition in the tibia, although supplementation with zinc carbonate hydroxide had no effect.

Table 2. Zinc deposition in the tissue of rats fed with experimental diets

Zinc added	Tissue zinc concentration (μg/g)				
	Plasma	Erythrocyte	Liver	Muscle	Tibia
None	0.29±0.08	2.51±0.11	20.8±1.4	8.09±0.59	13.2±1.6
Zinc carbonate hydroxide	0.29±0.07	2.41±0.10	22.3±0.7	8.55±0.41	11.8±0.4
ZRP	0.30±0.07	2.55±0.03	24.9±0.7	8.42±0.60	17.3±1.0

The above Tables demonstrates the unexpected results obtained by the experiments with zinc rich powder which is obtained by the process of extracting oyster with hot water, then adjusting the oyster residue to pH 2 to 4, filtering the residue extract and neutralizing the pH of the filtered extract as recited in the claims of the subject application.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001, of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: July 12, 2004

By : 松田芳和

Yoshikazu MATSUDA

J Nutr Sci Vitaminol, 49, 405–408, 2003

Zinc in Oysters (*Crassostrea gigas*): Chemical Characteristics and Action during In Vitro Digestion

Yoshikazu MATSUDA, Natsumi SUMIDA and Munehiro YOSHIDA

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Zinc in Oysters (*Crassostrea gigas*): Chemical Characteristics and Action during In Vitro Digestion

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(Received May 15, 2003)

Summary To obtain information on the luminal absorption of oyster zinc, the zinc action during an in vitro protease digestion of oysters was examined. More than 90% of the zinc rendered solute at pH 1.3 or 3.0 irrespective of the pepsin digestion. The solute zinc was partially re-precipitated by neutralization, and trypsin digestion did not render the re-precipitated zinc solute. When the pepsin digestion was performed at pH 5.0, the ratio of soluble zinc in trypsin digest decreased. When the trypsin digest was fractionated by Sephadex G-25, the zinc was eluted later than the peptide fragments. These results indicate that the pH of the stomach juice rather than peptides released by the digestion of oyster protein highly contributes to the oyster zinc in the small intestine becoming solute.

Key Words zinc, oyster, bioavailability, luminal absorption, in vitro digestion

Zinc is an essential trace element in human nutrition. The latest National Nutritional Survey has indicated that there is a suboptimal zinc status in the Japanese population (*1*). To improve zinc nutrition, effective utilization of several food sources with high zinc concentration is necessary. According to the Standard Tables of Food Composition in Japan, oysters contain zinc at a particularly high level (*2*), and so their use is expected in the zinc-enrichment of foods or the production of the zinc supplements.

It has been established that zinc in foods shows varying absorption rates due to various dietary factors including chemical characteristics (*3*). Even though oysters contain zinc at a high level, their contribution to zinc nutrition will remain unclear unless information on the absorption of oyster zinc is obtained.

Many approaches have been used to investigate zinc absorption in foods. The in vitro approach to zinc absorption has been performed by several researchers (*4–6*). In these reports, the solubility and chemical characteristics of zinc in an in vitro digest were used for an index of absorption. In the present study, to obtain information on the luminal absorption of oyster zinc, the chemical characteristics of oyster zinc and its action during an in vitro protease digestion of oysters were examined.

MATERIALS AND METHODS

Samples. Oysters (*Crassostrea gigas*) with a shell length of 10 to 15 cm were collected in the northern part of Hiroshima Bay in April 2002. Among them, 4 samples (zinc content: 123±19 μg/g) were selected randomly and homogenized with a homogenizer with a stainless steel blade (Cell Master CM-100, As-one Co., Osaka, Japan) individually.

Fractionation of oyster homogenate. Five grams of the whole oyster homogenate was diluted with 20 mL of Tris-HCl buffer (50 mM, pH 7.4) and centrifuged at 6,000×*g* for 30 min. After filtration with a membrane filter (0.20 μm), 100 μL of the soluble fraction obtained was analyzed using high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS). The HPLC system consisted of a CCPM-II multi-pump (Tosoh, Tokyo, Japan), an SD-8022 on-line degasser (Tosoh) and a column (4.6 mm i.d. ×250 mm) packed with a molecular exclusive resin (TSK-GEL 2000SWXL, Tosoh). The mobile phase was Tris-HCl buffer (50 mM, pH 7.4) or 0.1 M NaCl. Elution was performed isocratically at 1.0 mL/min. The eluate was successively monitored by a UV-8020 spectrophotometer (Tosoh) at an absorbance of 280 nm and by ICPMS (ICPM-8500, Shimadzu, Kyoto, Japan) at an ion intensity of *m/z* 66 derived from Zn^{66}. Another 5 mL of the soluble fraction was fractionated using a column (3.0 cm i.d. ×40 cm) packed with another molecular exclusive resin, Sephadex G-25. The samples were eluted with the Tris-HCl buffer or 0.1 M NaCl at a flow rate of 1.0 mL/min. Eluates were collected every 5 mL, the absorbance was measured at 280 nm and zinc content was determined.

In vitro protease digestion. One gram of the whole oyster homogenate was mixed with 50 mL of 0.1 M HCl containing 100 mg of a dried powder of porcine pepsin (1 : 10,000, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and incubated and shaken at 37°C for 3 h. The pH of this reaction mixture was 1.3. In another pepsin digestion test under a different pH condition, each homogenate was mixed with deionized water and adjusted to pH 3.0 or 5.0 by dropwise addition of 1 M

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HCl. In the case of the pepsin digestion at pH 1.3, the pH of the reaction mixture was adjusted to 7.4 by dropwise addition of 1 M NaOH after the digestion. After neutralization, 10 mg of a crystalline porcine trypsin (5, 600 USP trypsin unit/mg, Wako) was added to the mixture and incubated and shaken at 37°C for a further 16 h. At each step of the in vitro protease digestion, a portion of the reaction mixture was centrifuged at 6,000×*g* for 30 min. Zinc and nitrogen levels in the soluble fraction obtained were determined and their solubility was estimated as a percent of the contents in the soluble fraction to those in the whole mixture. In addition, the soluble fraction of the pepsin or pepsin-trypsin digest was fractionated by the Sephadex G-25 column (3.0 cm i.d. ×40 cm) with 0.1 M NaCl as elution solvent at a flow rate of 1.0 mL/min.

Analyses. One gram of the homogenate of the whole shelled oyster or each tissue was mixed with 10 mL of nitric acid (metal-free grade, Wako) and heated in a boiling water bath until the insoluble component disappeared. The protease digests were also rendered solute with nitric acid in a similar manner. Then, the acid digests were diluted with deionized water. The zinc level in the diluted acid digests and the eluates of the Sephadex G-25 chromatography was determined with an atomic absorption flame emission spectrophotometer (AA-6200, Shimadzu). The zinc analysis was verified using standard reference materials (RM 8414, bovine muscle powder, National Institute of Standard & Technology, USA). In addition, we confirmed that the zinc values in the oyster homogenate digested with nitric acid were coincident with those digested with nitric acid and perchloric acid. Nitrogen was determined by the Kjeldahl method.

Statistics. When necessary, analytical results were assessed by analysis of variance (ANOVA) followed by Fisher's PLSD test for multiple comparisons using a personal computer (iMac OS 8.6, Apple Computer, Cupertino, CA, USA) with the statistical analysis software package StatView ver. 5.0 (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Fractionation of whole homogenate

The solubility of zinc in the 20% oyster whole homogenate was 22.5%. Figure 1 shows the results of fractionation of the soluble fraction under 4 different gel chromatography systems. When the chromatography was performed using HPLC systems (Fig. 1a and b), zinc was co-eluted with high-molecular weight protein at a retention time that corresponded to a void volume irrespective of the kind of elution solvent. On the other hand, zinc was eluted into two peaks and the elution pattern of zinc was similar to that of the absorbance at 280 nm in the fractionation by Sephadex G-25 with Tris-HCl buffer (Fig. 1c). However, when 0.1 M NaCl was used as the elution solvent in the Sephadex G-25 column chromatography, zinc was eluted with a broad range and the elution pattern of zinc was not coincident with that of the absorbance at 280 nm (Fig. 1d).

Action of zinc during protease digestion

The solubility of zinc in each step of the protease digestion is summarized in Table 1. In the untreated oyster homogenate, more than 70% of the zinc was insoluble at pH 7.4. Addition of 0.1 M HCl to the homogenate made more than 90% of the zinc solute. Neutralization of the acidic pepsin digest caused partial insolubility of the zinc. However, digestion with protease did not affect the solubility of zinc. Even at the end of the in vitro protease digestion, about 30% of the zinc remained insoluble. On the other hand, the digestion with protease increased the soluble nitrogen. Nonethe-

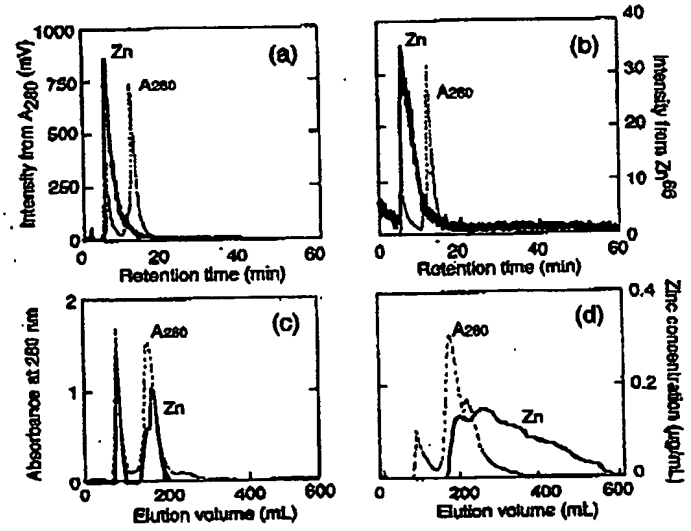

Fig. 1. Typical elution patterns in fractionation of soluble fractions of 20% whole homogenate of oyster prepared at pH 7.4. (a) fractionated by a TSK-GEL 2000SWXL with Tris-HCl buffer (pH 7.4, 50 mM) in HPLC-ICPMS system; (b) fractionated by a TSK-GEL 2000SWXL with 0.1 M NaCl in HPLC-ICPMS system; (c) fractionated by a Sephadex G-25 with Tris-HCl buffer (pH 7.4, 50 mM); (d) fractionated by a Sephadex G-25 with 0.1 M NaCl.

Table 1. Effect of in vitro protease digestion on solubilities of zinc and nitrogen in oysters.

Digestion step	Solubility (%)	
	Zinc	Nitrogen
2% homogenate in Tris-HCl buffer (pH 7.4)	26.5±5.9[a]	39.7±5.1[c]
2% homogenate in 0.1 M HCl (pH 1.3)	93.8±2.2[c]	13.6±4.5[a]
Pepsin digest (pH 1.3)	94.9±0.3[c]	25.4±3.9[b]
Neutralization of pepsin digest (pH 7.4)	68.1±1.0[b]	23.5±4.2[b]
Trypsin digest (pH 7.4)	68.6±4.2[b]	40.9±4.2[c]

Samples were collected in the northern part of Hiroshima Bay in April 2002. The weight of the shelled oyster was 19.7±3.9 g (means±SD, n=4). Values (means±SD, n=4) in the same column not sharing a common superscript letter differ significantly (p<0.05).

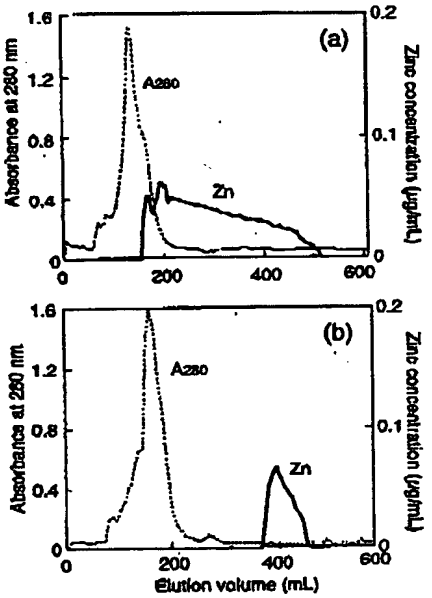

Fig. 2. Typical elution patterns in the fractionation of the soluble fraction of oyster protease digest by Sephadex G-25 with 0.1 M NaCl. (a) pepsin digest performed at pH 1.3 and neutralized to pH 7.4; (b) pepsin-trypsin digest.

less, about 60% of the oyster nitrogen still remained insoluble.

Figure 2 shows typical elution patterns by Sephadex G-25 gel chromatography of the soluble fraction of the protease digests. The elution pattern of zinc in the pepsin digest performed at pH 1.3 (Fig. 2a) was similar to that in the undigested homogenate (Fig. 1d). However, when trypsin-digestion was performed, the zinc was eluted later than the peptide fragment monitored by absorbance at 280 nm (Fig. 2b). When zinc chloride solution (10 μg zinc/mL) was fractionated in this chromatography system, zinc was eluted with a broad range at an elution volume of 500 to 750 mL (data not shown).

When pepsin digestion was performed at pH 3.0, the zinc solubility at each step was similar to that with pepsin digestion at pH 1.3 (Table 2). However, when pepsin

Table 2. Effect of pH condition during pepsin digestion on the solubility of zinc in oysters.

Digestion step	Solubility (%)		
	pH 1.3	pH 3.0	pH 5.0
Pepsin digest	93.7±3.6[b]	93.3±5.0[b]	60.3±4.8[a]
Trypsin digest (pH 7.4)	70.2±5.7[b]	62.3±6.8[b]	40.8±5.5[b]

Oyster samples were the same as those in Table 1. Values (means±SD, n=4) in the same row not sharing a common superscript letter differ significantly (p<0.05).

acted at pH 5.0, the zinc solubility, due to acidic pH, was incomplete; about 40% of the oyster zinc remained insoluble. This incomplete solubility in acidic pH influenced the zinc solubility at the end of trypsin digestion and more than half the oyster zinc remained insoluble.

DISCUSSION

As described in Table 1, more than 70% of the zinc was insoluble in the untreated oyster homogenate at pH 7.4. In the fractionation of the soluble fraction by HPLC with a molecular exclusive resin, zinc was co-eluted with the protein fraction (Fig. 1a and b). Thus, it is likely that the soluble zinc is mostly bound to protein. However, the affinity of zinc to protein may be mild because the elution pattern of zinc varied depending on the kind of resin or solvent used in the chromatography (Fig. 1c and d). Several metallothionein-like proteins containing zinc have been found in oysters (*7*, *8*). However, Fig. 1 shows the absence of such a specific zinc-containing component. Further examination is necessary in the search for a specific zinc-containing protein in oysters.

When the oyster homogenate was mixed with 0.1 M HCl, more than 90% of the zinc was rendered solute irrespective of the pepsin digestion. The solute zinc was partially re-precipitated by neutralization of the pepsin digest, but nearly 70% remained solute in the neutralized digest. Probably, most oyster zinc is present as a form tightly bound to oyster component(s), and is insoluble and biologically inactive. Diluted HCl, a main component in stomach juice, releases zinc from the component(s) as a bivalent zinc ion (Zn (II)) which is

highly soluble at a low pH. Because of the lower solubility of zinc hydroxides at pH 7 to 10 (9), a part of Zn (II) released by HCl could be re-precipitated by neutralization, but several parts remained soluble due to the formation of a complex with a protein or a peptide contained in the pepsin digest.

It has been believed that amino acids or peptides accelerate the luminal absorption of zinc due to the formation of a soluble complex with zinc (3). In addition, it has been reported that solubility of zinc in beef was accelerated by protease digestion (10). Therefore, it was expected that the release of peptides or amino acids by the protease from the oyster protein would increase the ratio of soluble zinc. However, as described in Table 1, pepsin or trypsin digestion did not increase the ratio of soluble zinc. When the trypsin digest was fractionated by Sephadex G-25, zinc was eluted later than the peptide fragments. Probably, the resin acted as a ligand for Zn (II). This implies that the affinity of Zn (II) with the peptide fragments released by the trypsin digestion is lower than that with the untreated protein.

When the pepsin digestion was performed at pH 5.0, the ratio of soluble zinc in the trypsin digest decreased (Table 2). These results indicate that the release of zinc by stomach juice highly contributes to the solubility of oyster zinc even in the small intestine; the pH of stomach juice rather than peptides released by the digestion of oyster protein is an important factor for zinc absorption.

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Influence of zinc from oyster extract on enzyme activity and tissue zinc concentrations = Comparison of other zinc sources =

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BIOMEDICAL RESEARCH ON TRACE ELEMENTS
Vol.14 No.4 2003 別刷

ORIGINAL ARTICLES

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Abstract

In a 4-week experiment, various sources of zinc were administered to Wistar rats. Twenty-four rats divided into 4 groups were given a zinc-deficient diet, oyster extract-added diet, zinc yeast-added diet and zinc sulfate-added diet, respectively. The zinc content of all the diets was adjusted to 5.5ppm except in the zinc-deficient diet. After 4 weeks, serum, liver, brain, thymus, spleen, kidney, tibia, muscle and testis were collected for zinc measurement, and serum alkaline phosphatase (ALP) activity and liver alcohol dehydrogenase activity (ADH) were measured. Regarding body weight gain and total food intake in the zinc sulfate group, an increased tendency was observed than in the other zinc-supplemented groups. The food efficiency was not changed among the 3 zinc-supplemented groups. Zinc concentration was significant increased in the brain in the zinc-supplemented group. In the zinc-deficiency group, serum ALP activity was significant decreased compared with the zinc-supplemented groups. ALP activity in the oyster-extract group was significant increased compared with the zinc-yeast group. In liver ADH activity, the zinc-deficiency group was also decreased compared with the zinc-supplemented groups. A significant increase in the oyster-extract group was recognized compared with the zinc-yeast and zinc sulfate group. These results suggested that different zinc sources in a diet do not influence zinc content in the liver, but ADH activity was influenced. A supplement of oyster extract was more useful in marginal zinc deficiency than the intake of zinc from other sources.

Key words: zinc, oyster extract, bioavailability, alkaline phosphatase, alcohol dehydrogenase

Introduction

Zinc is an essential trace element, and it is a factor in the formation of many enzymes. It participates in the metabolism of protein, lipids, carbohydrate, and nucleic acid, and it has broad functions such as growth, detoxication and an anti-oxidative effect. Dietary zinc deficiency has been shown in alopecia, dysgeusia, growth disease and damaged spermatogenesis [1]. However, despite being an essential element, zinc is a mineral which tends to be deficient. According to a national nutrition survey, it is reported that sufficiency rate of zinc was 80-90% of the nutritional requirement [2]. In addition, zinc intake is poor in the Japanese and US populations [3,4]. Generally, about 30% of zinc is absorbed after ingestion [5]. The absorbed rate of zinc changes in coexistence with organic substances. There has been a lot of research on interactions with zinc and dietary factors [6]. Fiber and phytic acid are known to inhibit absorption [7,8], whereas organic or amino acids are known to improve absorption [9]. They influence absorption by binding and chelating zinc, and prevent zinc from binding to non-absorbable dietary compounds. In this way, the utilization efficiency in the organism changes with the form of zinc and coexisting substances. Ingesting substances with a positive effect on zinc absorption is important nutritionally. Oysters (*Crassostrea gigas*) are known to contain a

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large amount of zinc, and various nutrients, such as glycogen and amino acids, are also present. The characteristics and physiological availability of zinc in raw oysters have been examined extensively [10]. In addition, hot water extract from oysters has physiological availability [11-14]. However, in raw oysters, water-soluble zinc is only 10-20%. Most zinc is insoluble, and a problem therefore exists for its nutritional availability.

We therefore examined isolation and concentration methods for insoluble zinc contained in oysters. When it was administered to rats, it was accumulated by the tissue of the liver and tibia. Extracted zinc from oysters may be absorbed effectively. In this experiment, oyster extract and other zinc sources were administered to rats, and the zinc content in various organs was measured. The activity of alcohol dehydrogenase (ADH) in the liver and alkaline phosphatase (ALP) in serum were also measured.

Materials and methods

Animals and feeding

Commercial male SPF/VAF Crj: Wistar rats (4 weeks of age, Charles River Japan Inc.) were used. They were given commercial pellets (CE-2, Japan Clea Co., Ltd.) and tap water *ad libitum* for 1 week. They were 5 weeks of age and weighed 90-100g at the start of the experiment. The animals received proper care and maintenance in accordance with institutional guidelines. They were housed in a room with a temperature of 23 ± 2℃, relative humidity of 60 ± 10% and lighting for 12h a day (lighting from 6:00 to 18:00) throughout the experiment. They were housed in groups of 3 in stainless steel mesh-bottomed cages. The composition of the zinc-deficient diet (Oriental Yeast, Co., Ltd) was 20% spray-dried egg white solid, 63.7% dextrose, 10% corn oil, 2% non-nutritive cellulose fiber, 3.13% mineral mix and 1.17% vitamin mix. The mineral mix contained (g/100g) : NaCl 17.75, K_2HPO_4 34.16, $MgSO_4$ 5.28, $CaHPO_4$ 7.96, $CaCO_3$ 31.79, Fe-Citrate 2.91, KI 0.084, $MnSO_4 \cdot 4 \sim 5H_2O$ 0.028, $CuSO_4 \cdot 5H_2O$ 0.032 and $CoCl_2 \cdot 6H_2O$ 0.006. The vitamin mix contained (per 100g) : retinol 85,470IU, cholecalciferol 10,680IU, alpha-tocopherol 940mg, vitamin K_3 2.82mg, thiamine 85.5mg, riboflavin 51.3mg, pyridoxine 34.2mg, cyanocobalamin 0.17mg, biotin 34.2mg, folic acid 4.27mg, calcium pantothenate 136.8mg, nicotinic acid 213.7mg and choline chloride 12.8g. These vitamins were adjusted to 100g with cellulose powder. The zinc content of this diet was less than 0.6ppm. The oyster extract (Japan Clinic Co., Ltd.), zinc yeast (Mineral yeast ZN, purchased Oriental Yeast, Co., Ltd.) and reagent zinc sulfate (Wako Pure Chemical Industries) were used as the sources of zinc. The composition of the oyster extract is shown in Table 1. These test materials were each mixed with the zinc-deficient diet. The zinc content of all diets was adjusted to 5.5ppm except the zinc-deficient diet. The animals were divided into 4 groups of 6 (zinc-deficient diet, oyster extract-added diet, zinc yeast-added diet and zinc sulfate-added diet). The diet and drinking water (distilled water) were given *ad libitum*. They were kept for 4 weeks, and their food intake and body weight were measured every 2 or 3 days. In addition, the feeding efficiency was estimated by body weight gain divided by food consumption.

Table 1. Composition of oyster extract

Components	Contents (dry weight)	
Protein	28.7	%
Fat	2.0	%
Ash	17.8	%
Taurine	5.46	%
Glycogen	34.5	%
Zinc	152.9	mg/100g
Copper	9.66	mg/100g
Iron	14.8	mg/100g
Calcium	214	mg/100g
Magnesium	409	mg/100g

Oyster extract was manufactured by Japan Clinic Co., Ltd.

Experimental procedure

After 4 weeks, the rats were anesthetized with ether, and whole blood samples were obtained from the abdominal aorta. They were killed by exsanguination, and the liver was removed and immediately refluxed with cold physiological saline. It was weighed before and after the reflux. The collected whole blood was centrifuged at 3000rpm, 10min, 4℃ and serum obtained. Enzyme activity and zinc content were then measured. In the other tissues, the brain, thymus, spleen, kidney, tibia, muscle and testis were collected for zinc measurement. The brain was separated into the cerebrum and other sections, and the muscle was used from around the tibia.

Measurement of zinc in tissues

The zinc concentration in the serum samples was directly measured by the furnace method using an atomic absorption spectrophotometer (AA-6800, Shimazu Corporation). Other tissues were homogenized with Potter's

homogenizer, and digested using a nitric acid wet digestion procedure. The flame suction method was used with an atomic absorption spectrophotometer. Certified mineral reference solutions (Wako Pure Chemical Inc.) were used as standards.

Measurement of ALP

ALP activity in serum was measured using a commercial kit (ALP K-test Wako : Wako pure Chemical Inc.). The phenylphosphate substrate method was used [15].

Measurement of ADH

Ethanol oxidation was performed in Tris-HCl buffer at 30℃. Coenzyme concentrations were NAD^+ in oxidation assays and this was determined by monitoring the change in NADH concentration with a Shimazu UVPC-2700 spectrophotometer using an NADH molar absorption coefficient of $6300M^{-1}cm^{-1}$ at 340nm. 0.5g of liver was added to cold 50mM Tris-HCl buffer containing 0.1% bovine serum albumin pH 8.5, and adjusted to 5ml. Homogenization was carried out in a Potter's homogenizer. After centrifugation (3000 rpm, 10min, 4℃), the supernatant was obtained as a crude extract for measuring the ADH activity. 0.8ml Tris-HCl buffer, 1ml 0.3mM NAD^+, 1ml 0.3M ethanol and 0.2ml crude extract were mixed, and the change of absorbance over one minute was recorded. The slope of the early reaction stage was determined by the increased rate of absorbance. The enzyme activity that reduced 1 μmol NAD^+ over one minute was expressed as 1 unit.

Statistical procedures

The experimental data are presented as the mean ± standard deviation. After the variance of values to be compared had been assayed by F-test, the results were analyzed by Student's t-test; the significant difference of data not showing equal variance was judged by Welch's t-test. Calculations were performed using Microsoft Excel 2000, with $p<0.05$ being defined as significant.

Results

In the rats of the zinc-deficiency group, severe signs of zinc deficiency such as growth retardation, alopecia and dermatitis were observed in all individuals. The change in body weight is shown in Fig.1. The mean body weight of the zinc-deficiency group, zinc-yeast group, zinc sulfate group and oyster-extract group at the start of the experiment was 99.2 ± 7.0 g, 77.9 ± 6.2 g, 86.4 ± 3.2g and 87.5 ± 1.9g, respectively. At the end of the experiment, it was 161.7 ± 48.5g, 230 ± 9.7g, 285 ± 28g and 259 ± 20.6g, respectively. The body weight of the zinc-deficiency group was significantly reduced in comparison with the other groups, and remarkable growth retardation was noted. In the 3 groups except the deficiency group, there was increased tendency in the zinc sulfate group in comparison with the other 2 groups. No other indications were noted, and steady development was observed. Fig.2 shows the food intake: in the zinc-deficiency group, the intake decreased remarkably with the food intake being almost proportional to the body weight. The total food intake, total zinc intake (μg), body weight

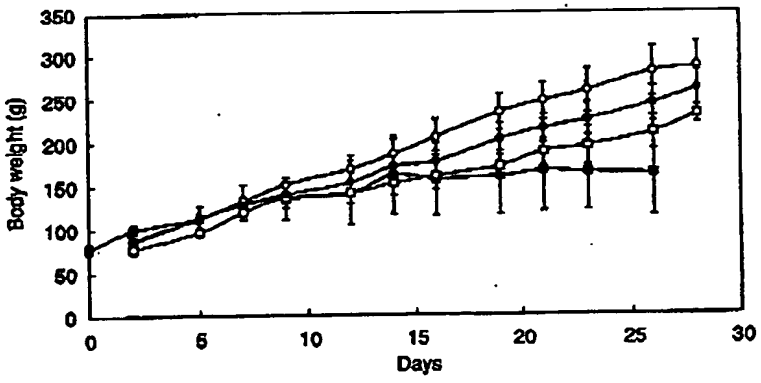

Fig.1 Changes in body weight in Wistar rat fed zinc deficient and zinc supplement diet
Markers and vertical bars represent means and standard deviation range, respectively.
■: Zinc deficiency group; □: Zinc yeast supplemented group; ●: Oyster extract supplemented group; ○: Zinc sulfate supplemented group.
All diets were given for 9 weeks from 5 weeks of age.

gain and food efficiency are shown in Table 2. The total food intake, body weight gain and total zinc intake increased by order of the zinc-deficiency group < oyster extract group < zinc-yeast group < zinc sulfate group. The body weight gain of the zinc-supplemented groups was significantly different from the zinc-deficiency group. Regarding feed efficiency, a lower tendency was observed in the zinc-deficiency group, and this difference was barely noted in the other groups. Table 3 shows each organ weight. In the zinc-yeast group, the organ weights were not significantly different from the zinc-deficiency group. In the zinc sulfate group, when compared with the zinc deficiency group, there were significant differences except for the brain, tibia and testis. The

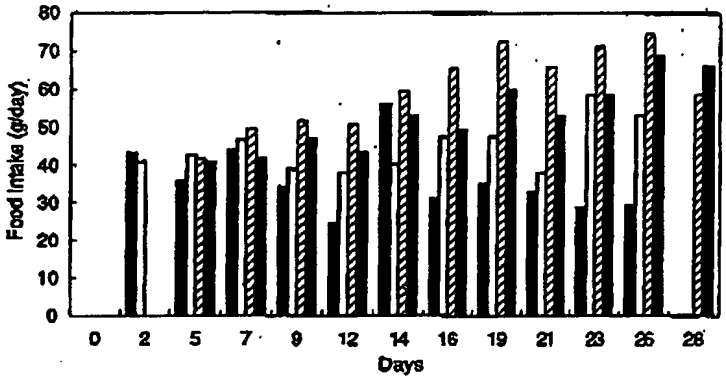

Fig.2 Changes in food intake in Wistar rat fed zinc deficient and zinc supplement diet
■: Zinc deficiency group; □: Zinc yeast supplemented group; ▨: Oyster extract supplemented group; ■: Zinc sulfate supplemented group.
Food intake was measured every 2 or 3 days per cage.

Table 2. Total food intake, zinc intake, body weight gain and food efficiency

	Total food intake (g)[1]	Total zinc intake (mg)[2]	Body weight gain (g)[3]	Food efficiency (%)[4]
Zinc deficient	305	183	83.8±42.4	27.5
Zinc yeast	388	2135	152.1±6.6**	39.2
Zinc sulfate	520	2862	199.4±25.6**	38.3
Oyster extract	458	2518	172.1±21.3**	37.6

1: Total food intake was calculated by the sum of food intake for 9 weeks from 5 weeks of age.
2: Zinc concentrations in zinc deficient diet and other diet were 0.6ppm and 5.5ppm, respectively.
3: Values are mean±SD. Body weight gain was calculated by body weight of 9 weeks minus 5 weeks. **: p<0.01: compared with the zinc deficiency group.
4: Food efficiency was calculated by body weight gain / total food intake×100.

Table 3. Body weight and tissue wet weight of zinc deficiency and administration of various zinc sources

Tissue wet weight (g)	Zinc deficiency	Zinc yeast	Zinc sulfate	Oyster extract
Body weight	162±48	230±10	286±28dy	260±21d
Liver	4.63±1.47	6.27±0.55	7.90±0.60dy	7.16±0.33d
Brain	1.76±0.13	1.81±0.05	1.85±0.02	1.83±0.10
Thymus	0.31±0.09	0.43±0.07	0.69±0.07dy	0.57±0.18
Spleen	0.41±0.09	0.57±0.09	0.68±0.10d	0.63±0.09d
Kidney	1.60±0.10	1.65±0.16	2.27±0.09ddyyo	2.01±0.10ddy
Tibia	0.31±0.08	0.39±0.03	0.41±0.03	0.42±0.11
Testis	2.60±0.71	2.78±0.30	3.01±0.23	2.94±0.33

Values are mean±S.D.
dd: p<0.01, d: p<0.05, compared with the zinc deficiency group.
yy: p<0.01, y: p<0.05, compared with the zinc yeast group.
o: p<0.05, compared with the oyster extract group.

Table 4. Rate of body weight of zinc deficiency and administration of various zinc sources

Rate of body weight (%)	Zinc deficiency	Zinc yeast	Zinc sulfate	Oyster extract
Liver	2.85±0.16	2.74±0.23	2.77±0.06	2.77±0.18
Brain	1.14±0.30	0.87±0.01	0.65±0.06	0.70±0.02
Thymus	0.189±6	0.189±23	0.243±20dd,y	0.217±50
Spleen	0.258±34	0.252±29	0.238±15	0.240±20
Kidney	0.84±0.05	0.79±0.07	0.80±0.08	0.78±0.02dd
Tibia	0.191±6	0.163±7dd	0.144±10dd	0.161±28
Testis	1.62±0.05	1.21±0.08dd	1.06±0.03dd,y	1.13±0.04dd

Rate of body weight were calculated by tissue weight / body weight × 100
Values are mean±SD.
dd: p<0.01, compared with the zinc deficiency group.
y: p<0.05, compared with the zinc yeast group.

Table 5. Zinc concentrations in tissue

	Tissue zinc concentration (μg/g wet weight)			
	Zinc deficiency	Zinc yeast	Zinc sulfate	Oyster extract
Liver	49.7±8.8	42.2±6.9	48.0±2.4	43.7±6.9
Brain	11.6±0.1	13.2±0.8d	14.1±1.2d	13.2±0.5dd
Thymus	32.2±4.1	30.3±1.1	31.2±5.1	28.9±2.9
Spleen	27.7±1.7	25.8±2.0	26.1±0.7	27.5±0.7
Kidney	30.5±2.0	28.8±2.1	27.3±1.4	27.9±1.7
Tibia	58.4±8.3	61.9±3.2	69.5±4.4	62.9±3.4
Muscle	21.5±5.3	21.4±0.7	19.3±2.1	20.1±6.6
Testis	21.5±2.3	24.0±2.8	22.0±3.1	25.5±2.0
Erythrocyte	9.4±1.4	10.7±0.6	9.8±0.6	9.0±1.0
Serum	0.7±0.28	0.97±0.07	1.09±0.09	1.09±0.09

Values are mean±SD.
dd: p<0.01, d: p<0.05, compared with the zinc deficiency group.

increases of the liver, thymus, spleen and kidney were 171%, 223%, 166% and 142%, respectively. For the liver, thymus and kidney, there were increases of 126%, 160% and 138% against the zinc-yeast group. For the kidney, there was a 113% increase over the oyster-extract group. In the oyster-extract group, the liver, spleen and tibia increased significantly over the zinc-deficiency group by 155%, 154% and 126%. For the kidney, an increase of 122% was seen in the zinc-yeast group. Table 4 showed the body weight rate of each organ. For the thymus, an increase of 129% was seen in the zinc sulfate group against the zinc-deficiency group and zinc-yeast group. For the oyster-extract group, an increased tendency was seen over the zinc-deficiency group. The kidney, tibia and testis decreased as a whole against the zinc-deficiency group. Table 5 shows the zinc content of each tissue. In the brain, the zinc content increased significantly in all groups against the zinc-deficiency group. In the other tissues, no significant difference was recognized. However, in the zinc-supplemented groups, for the tibia, testis

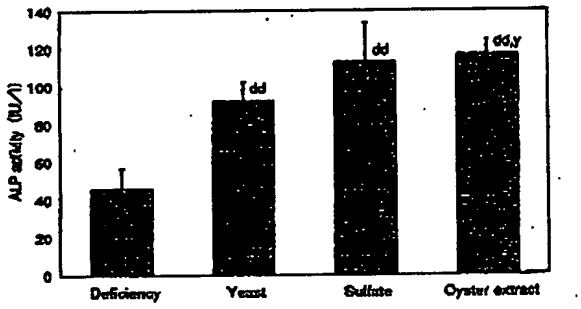

Fig.3 Effect of dietary zinc on serum alkaline phosphatase (ALP) activity

Rectangle and vertical bars represent means and standard deviation range, respectively.
dd: p<0.01, compared with the zinc deficiency group.
y: p<0.05, compared with the zinc yeast group.

and serum, an increased tendency was seen against the zinc-deficiency group. Fig. 3 shows the ALP activity in the serum. The ALP activity of the zinc-deficiency group was 45.6±11.1IU/l, and decreased in significance compared with the other groups. In the zinc supplemented-

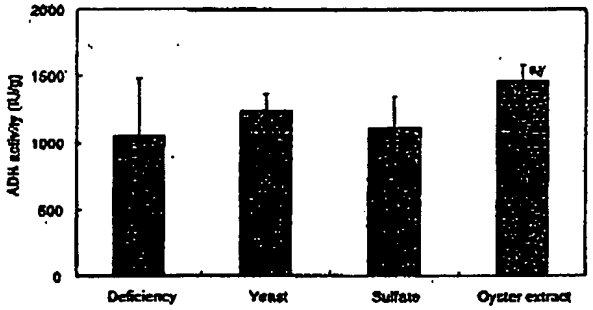

Fig.4 Effect of dietary zinc on alcohol dehydrogenase (ADH) activity in 1g of liver

Rectangle and vertical bars represent means and standard deviation range, respectively.
s: $p<0.05$, compared with the zinc sulfate group.
y: $p<0.05$, compared with the zinc yeast group.

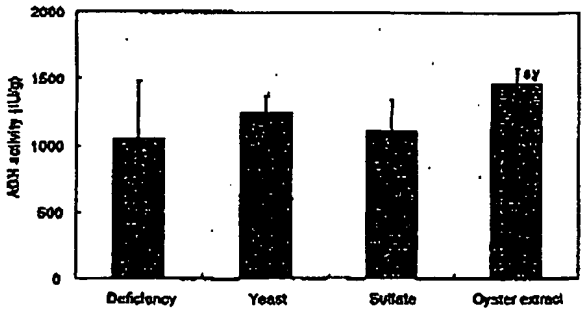

Fig.5 Effect of dietary zinc on alcohol dehydrogenase (ADH) activity in whole liver

Rectangle and vertical bars represent means and standard deviation range, respectively.
dd: $p<0.01$, d: $p<0.05$ compared with the zinc deficiency group.
y: $p<0.05$, compared with the zinc yeast group.
s: $p<0.05$, compared with the zinc sulfate group.

groups, the enzyme activity was in the order of oyster extract (116.8 ± 7.9IU/l)> zinc sulfate (112.4 ± 20.9IU/l)> zinc yeast (92.3 ± 9.9IU/l). In the oyster-extract group, significantly higher activity was seen compared with the zinc-yeast group, although it was not significant for the zinc-sulfate group. Fig. 4 shows the ADH activity in the 1g liver. The ADH activity of the zinc-deficiency group was 1049 ± 430IU/g, almost identical to the 1109 ± 235IU/g of the zinc sulfate group. The zinc-yeast group was 1239 ± 121IU/g, higher than in the groups above. In the oyster-extract group, it was 1468 ± 112IU/g, significantly higher than the zinc-yeast group and zinc sulfate group. Regarding the zinc-deficiency group, the standard deviation was high, but no significant difference was noted. Fig. 5 shows the ADH activity of the whole liver and a similar result was obtained. However, a larger significant difference was noted. The zinc-deficiency group decreased in significance for the oyster-extract group and zinc sulfate group, and the oyster-extract group increased in significance over all the other groups.

Discussion

Zinc is absorbed in the small intestine, with the main absorption site being the duodenum and intestinum jejunum. Generally, the absorption rate of zinc is about 30% of the intake. Zinc absorption is influenced by the state of the coexistence substance as described previously. Zinc is classified as inorganic zinc, such as sulfate, chloride and oxide, and organic zinc, such as gluconate and anino acid complexes. In the USA, zinc gluconate is a highly bioavailable over-the-counter dietary zinc supplement and it has been suggested to cause less stomach irritation than zinc sulfate. In this study, zinc sulfate was used as the inorganic zinc source, being generally recognized as having relatively high bioavailability. For the organic zinc, oyster extract was used. Zinc yeast is widely used as a zinc supplement in Japan, therefore, as a comparison control, zinc yeast was also used. It is possible that the production method of zinc yeast adds inorganic zinc to yeast, therefore, whether zinc is organic or inorganic is unclear in zinc yeast. The extract and yeast used as zinc sources in this study contain components other than zinc and the influence of these components must be considered. There are numerous investigations on the availability of organic and/or inorganic zinc in different species under various conditions and with different results. For example, Cheng [16] reported that after administering a supplement to pigs for about 30 days, the bioavailability of zinc sulfate was higher than that of zinc lysine when food with a high lysine content (1.1%) was given. In contrast, if food with a low lysine content (0.8%) was supplemented with zinc lysine, the bioavailability was higher than that of zinc sulfate. Wichert [17] reported that a zinc-methionine complex was more bioavailable than zinc sulfate as measured by the zinc content of the tibia of chicks. In contrast, Ley [18] reported no difference in bioavailability between an inorganic zinc and a chelated mineral compound in horses supplemented for at least 30 days. Danek [19] measured the serum response in a long-term experiment (30 days) using different concentrations of zinc sulfate, and also reported the high bioavailability of zinc sulfate. Schryver

[20] reported increased zinc concentration in serum after long-term supplementation with zinc oxide. From these diverse findings, the effectiveness of different zinc sources has not been confirmed.

In the zinc-deficiency group, severe signs of zinc deficiency were observed and decreasing ALP activity was noted. In the serum zinc level, a tendency to decrease was observed ($p=0.08$) in the zinc-deficient group. These were considered to demonstrate zinc deficiency. A tendency toward decreased body weight was observed, except in the zinc-deficiency group. No group with sufficient zinc was examined in this study. However, according to the catalog of experimental animals of Charles River Japan Inc., standard body weight of 9-week-old male Wistar rats is about 320g. Moreover, the results of our preliminary study showed that rats fed a zinc-deficient diet containing 40ppm zinc (unpublished data) demonstrated similar development under the same conditions as in the study, and there was a possible marginal zinc deficiency. However, in the zinc-supplemented group, the development was steady compared with the zinc-deficiency group. It was suggested that this depended on the source of zinc. The tissue weight of the zinc sulfate group increased compared with the other groups. It is suggested that more food was consumed compared with the other groups. Regarding food intake and body weight gain, an increased tendency was seen in the zinc sulfate group, but the food efficiency did not differ from the other zinc-administered group. The zinc consumption of the oyster-extract group during the experimental period was about 0.9 times that of the zinc sulfate group, suggesting that zinc sulfate may not be utilized effectively. In the zinc yeast group, zinc consumption was lower than other zinc-administered groups. It was about 0.74 times that of zinc sulfate group, and 0.84 times that of oyster extract group. Zinc from zinc yeast may be utilized effectively.

Organic substances such as amino acids were contained in the other zinc-administered groups in addition to zinc. Histidine, cysteine and taurine are known to be amino acids influencing the absorption of zinc. Zinc-amino acid chelate appeared to have the most important influence on zinc absorption. Amino acids may increase bioavailability by removing chelated zinc from dietary zinc-binding constituents such as phytic acid. Histidine has a strong zinc-chelating effect, and clinical investigations in humans have shown the positive effect of histidine on zinc absorption as measured by the increase of plasma zinc [21]. Cysteine had a stimulatory action on post-intestinal zinc absorption, distinct from the effects of histidine [22]. Cysteine infusion in rats was found to increase plasma zinc levels [23]. These amino acids are contained in oyster extract, and it is probable that they influence absorption. However, only about 0.4% of oyster extract was added to the diet, and therefore the content was very little quantitatively. Increasing the dosage of the oyster extract may influence the absorption. Taurine was included as an amino acid of biological importance, but without known chelation effects. There are few reports about the relationship between taurine and zinc absorption. Harraki reported the taurine stimulation of zinc absorption in human fibroblasts [24]. It has also been reported that membrane stability was enhanced by the combination of taurine and zinc [25]. The oyster extract contained a high amount of taurine and a positive effect on zinc absorption may have been seen.

On the other hand, zinc plays an important role as an activation center for enzymes, and a number of zinc enzymes exist. The activity of ALP and ADH was measured to examine whether oyster extract influenced the utilization of enzymes in addition to zinc absorption. ALP hydrolyzes most phosphoric monoester bonding, and it is an enzyme generating inorganic phosphate. Clinically, the elevation is doubted in liver disease, biliary tract disease and bone disease. ADH catalyzes oxidation-reduction between alcohol and aldehyde. Both enzymes have zinc in their active center, and require zinc. Four zinc atoms connect ALP to the enzyme molecule, 2 participating in the active center, and the remaining 2 participating in the stabilization of the steric structure of the enzyme molecule. ADH contains 2 atoms of zinc per subunit. When the enzyme is chelated, activity is lost. In animals and humans, there are numerous reports that this enzyme activity decreases with zinc deficiency. In particular, ALP is used with serum zinc concentration as an indicator to diagnose zinc deficiency in humans [26,27]. The activity of ALP and ADH increased in significance in the oyster-extract group compared with the other groups. Assuming that the serum ALP activity was indicative of the zinc-deficient status, zinc in the oyster extract was effectively replete compared with other zinc sources. It has been suggested that zinc from the oyster extract is used for the effective synthesis of enzymes, or that enzyme activity increased by other components contained in the oyster extract. Plapp reported that the amino group was important in ADH activation with zinc [28]. Moreover, there are reports that taurine and carbohydrate were associated with ADH activation [29,30]. Thus, many inter-

actions between ADH activity and various substances have been considered. The oyster extract contained a significant amount of these substances, such as zinc, taurine and glycogen that may interact for enzyme activity.

In conclusion, no significant change in zinc content in various tissues was noted. However, ALP and ADH activity improved in the oyster-extract group compared with the other groups. The bioavailability of the oyster-extract group is superior when food efficiency and enzyme activities are considered. A different zinc source in a diet does not influence the zinc content of the liver, but ADH activity is influenced.

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Preparation of Zinc-Rich Powder from Oysters and Evaluation of Its Bioavailability

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BIOMEDICAL RESEARCH ON TRACE ELEMENTS
Vol.14 No.4 2003 別刷

ORIGINAL ARTICLES

Preparation of Zinc-Rich Powder from Oysters and Evaluation of Its Bioavailability

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Abstract

Hot water extract of oyster is used as a raw material of several nutritional supplements and oyster sauce. However, most of zinc in oysters is difficult to be recovered by the hot water extract method. In this study, we examined a zinc-rich powder fraction (ZRP) from oyster scraps in preparation of the oyster extract, and evaluated its bioavailability by animal assay and an *in vitro* digestion test. In preparation of ZRP, extraction with 0.1M HCl was performed for the oyster scraps. After neutralization of the acid extract, the precipitation was collected, freeze-dried and used as ZRP. The zinc content of ZRP was 76 mg/g in the dry base. About 70% of the zinc in the scraps was recovered into ZRP. In the evaluation of bioavailability, male weanling Wistar rats were pair-fed a low zinc-based diet (zinc content, 1.41 μg/g) or a basal diet supplemented with zinc carbonate hydroxide or ZRP (zinc content, 5 μg/g) for 4 weeks. The zinc content of the tibia of the rats supplemented with ZRP was significantly higher than that of the rats supplemented with zinc carbonate hydroxide. In an *in vitro* digestion test, when the zinc-supplemented diet was digested with trypsin, the zinc in ZRP was solubilized more rapidly than that in zinc carbonate hydroxide. These results indicate that ZRP contains a high level of zinc in a highly available form. Accordingly, ZRP can be used as a zinc supplement foodstuff.

Key words: zinc, oyster, bioavailability, zinc supplement foodstuff.

INTRODUCTION

Zinc is an essential trace element in human nutrition. The latest National Nutritional Survey indicated that there is a sub-optimal zinc level in the Japanese population [1]. To improve zinc nutrition, the effective utilization of several food sources containing a high zinc concentration is necessary. According to the Standard Tables of Food Composition in Japan, oysters contain zinc at a particularly high level [2], and so their use is expected in the zinc enrichment of foods or the production of the zinc supplements.

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In Japan, hot water extract of oyster (OE) is utilized as a raw material of several supplements and oyster sauce. However, much of the zinc in oysters is insoluble in water at a neutral pH [3], and cannot be extracted with hot water. This means that the zinc concentration in OE is too low to use as a zinc supplement. Although most of the zinc remains in the oyster after hot water extraction, the oyster is scrapped as waste without recovering the zinc.

In this study, we used the oysters scrapped in OE production to recover zinc as a form of zinc-rich powder (ZRP). In addition, we studied the bioavailability of zinc in the powder by *in vivo* animal assay and *in vitro* digestion test.

MATERIALS AND METHODS

Samples. Hot air-dried samples of oysters scrapped

in OE production were kindly supplied by Japan Clinic Co. (Kyoto, Japan) and ground using a mill (Grindomix GM200, Retsch GmbH & Co, Haan, Germany). Analysis using an atomic absorption flame emission spectrophotometer (AA-6200, Shimadzu, Kyoto, Japan) showed that the zinc content in the samples was 3.07 mg/g.

Preparation of ZRP. The dried powder of the oyster scraps was mixed with 20 volumes of 0.1 M HCl. The mixture remained at room temperature with gently shaking for 16 h, and was then centrifuged at 750 x g for 20 min. The supernatant obtained was adjusted to pH 7.0 by the dropwise addition of 1.0 M NaOH. The precipitation formed after neutralization was collected by centrifugation at 6,000 x g for 20 min. The precipitation was freeze-dried and used as ZRP from oysters.

Animal feeding. The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the Guide for the Care and Use of Experimental Animals of the Prime Minister's Office of Japan. Four-week-old male Wistar rats (n=18), weighing 40 to 50 g each, were housed in stainless steel wire mesh hanging cages at a temperature of 22 to 24 ℃ and a humidity of 60% in a room with a controlled 12 h light (08:00 to 20:00) and dark cycle. The rats were given deionized water *ad libitum* and pair-fed the diet throughout the experimental period.

They were randomly divided into 3 groups. One group was fed a casein-based low zinc diet (referred to as the basal diet). The other two groups were fed the experimental diets, supplementing the basal diet with 5 μg/g of zinc as either zinc carbonate hydroxide or ZRP. The composition of the basal diet was (%): casein, 20.0; sucrose, 15.0; α-starch, 52.0; soybean oil, 8.0; AIN93G mineral mixture (except for zinc carbonate hydroxide), 3.5; AIN93G vitamin mixture, 1.0; choline bitartrate, 0.2; DL-methionine, 0.3; and cellulose powder, 2.0. Analysis showed that the basal diet contained 1.41 μg/g of zinc. After feeding for 4 weeks, the blood, liver, muscles and tibia were isolated, washed, blotted and weighed. The Blood was collected by heart puncture under the anesthetizing with diethylether.

In vitro digestion. Ten grams of each zinc-supplemented diet were mixed with 50 ml of 0.1N HCl containing 300 mg of a dried powder of porcine pepsin (1:10,000, Wako Pure Chemical Industries, Osaka) and incubated with shaking at 37 ℃ for 3 h. The pH of this reaction mixture was 1.3. After pepsin digestion, the pH of the reaction mixture was adjusted to 7.4 by the dropwise addition of 1M NaOH. Then, 30 mg of crystalline porcine trypsin (5,600 USP trypsin unit/mg, Wako) was added to the mixture and incubated with shaking at 37 ℃ for a further 16 h. At each step of the *in vitro* protease digestion, a portion of the reaction mixture was centrifuged at 6,000 x g for 30 min.

Zinc in each soluble fraction obtained was directly determined with an atomic absorption flame emission spectrophotometer and the solubility was estimated as a percent of the contents of the soluble fraction to that in the whole mixture.

Assays. The chemical composition of ZRP was analyzed as follows. Moisture was measured by oven drying at 105 ℃. Crude protein content was calculated from Kjeldahl nitrogen. Crude lipid was extracted by diethylether with a Soxhlet extractor after a hydrolysis with 35% HCl, and then weighed. Ash was weighed after direct incineration at 550 ℃. After a simultaneous digestion with α-amylase, amyloglucosidase and protease, the undigestible fraction formed was collected, dried and incinerated. Dietary fiber in the ZRP was calculated as a difference between the dry weight and ash content of the undigestible fraction as described by Prosky [4]. Carbohydrate content was calculated from the following equation: 100 - (moisture + crude protein + crude lipid + ash + dietary fiber). Glycogen was extracted with hot water and determined with anthrone [5].

The ash resulting from the direct incineration of ZRP was dissolved in 1% HCl and subjected to mineral analysis. Sodium, potassium, magnesium, zinc, copper and manganese levels were determined using an atomic absorption flame emission spectrophotometer [6]. Phosphorous was determined colorimetrically using molybdate and vanadinate [6]. Iron was determined with 1,10-phenanthrolinium chloride [6]. Calcium was determined by titration using oxalic acid and potassium permanganate [6].

The rat tissue was mixed with HNO_3 and heated in a boiling water bath until the insoluble components disappeared. The acid digests were then diluted with deionized water. The zinc level in the diluted acid digests was determined with an atomic absorption flame emission spectrophotometer. The zinc analysis was verified using standard reference materials (RM 8414, bovine muscle powder, National Institute of Standard & Technology, USA).

Statistics. Data obtained from the animal experiments were assessed by analysis of variance (ANOVA) followed by a PLSD test for multiple comparisons using a personal computer (eMac, Apple Computer, Cupertino, CA, USA)

with the statistical analysis software package StatView ver. 5.0 (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Recovery of zinc. More than 95% of the zinc in the oyster scraps was extracted by 0.1 M HCl. Neutralization of the extract with NaOH formed a precipitate and 70 to 80% of the zinc extracted was recovered in the precipitate. Thus, about 70% of the zinc in the oyster scraps was recovered in the precipitate.

Chemical composition. Freeze-dried powder of the precipitate, that is, the oyster ZRP, was gray in color with a slight fishy odor. Table 1 shows the major components of ZRP, which was mainly composed of Kjeldahl nitrogen and ash.

Table 2 describes several minerals in ZRP, which contained zinc at a level of 76 mg/g, 25 times higher than the original oyster scraps. The levels of sodium, calcium and phosphorous were comparable to zinc, but other minerals were less than 10 mg/g.

In vitro digestion. Table 3 shows the ratio of soluble zinc in each step of the *in vitro* protease digestion of the zinc-supplemented diets. At a neutral pH, the zinc solubility of the experimental diets was less than 30%, irrespective of the zinc source. Most of the dietary zinc was solubilized by 0.1 M HCl regardless of pepsin digestion, but was re-precipitated by neutralization.

Figure 1 shows the release of soluble zinc from each zinc-supplemented diet during trypsin digestion. The zinc in both diets was completely solubilized by trypsin digestion for 16 h. However, the speed of release varied with the zinc source, that is, the zinc in ZRP was more

Table 1. Composition of zinc-rich powder of oysters

	Content (%)
Moisture	2.7
Crude protein	43.4
Crude lipids	1.2
Ash	40.1
Dietary fiber	1.5
Carbohydrate	11.1
Glycogen	6.1

Table 2. Mineral content of zinc-rich powder of oysters

Minerals	Content (mg/g)
Zinc	76.0
Sodium	62.5
Phosphorus	46.5
Iron	4.70
Calcium	22.4
Potassium	4.00
Magnesium	4.05
Copper	2.78
Manganese	1.26

Table 3. Solubility of zinc contained in experimental diets

Solvent or treatment	Solubility of zinc added (%)[1]	
	Zinc carbonate hydroxide	Zinc-rich powder of oysters
Water[2]	7.6±2.4	7.0±2.6
0.1M NaCl[2]	13.3±3.1	14.4±4.6
50 mM, Tris-HCl buffer (pH 7.4)[2]	27.1±3.5	23.3±4.5
0.1 M HCl[2]	89.4±5.9	91.6±4.7
Pepsin digest (pH 1.3)[3]	89.1±6.1	92.4±3.8
Pepsin digest (pH 7.4)[3]	17.5±4.5	21.3±5.1

[1] Values are means ± SD for 4 trials. Significant difference ($p<0.05$) was not observed between zinc carbonate hydroxide and zinc-rich powder of oysters in all the solvents or treatments.
[2] Ten grams of each zinc-supplemented diet were mixed with 50 ml of each solvent.
[3] Ten grams of each zinc-supplemented diet were mixed with 0.1N HCl containing 300 mg of a dried powder of porcine pepsin and incubated with shaking at 37 ℃ for 3 h. The pH of this reaction mixture was 1.3. After pepsin digestion, the pH of the reaction mixture was adjusted to 7.4 by the dropwise addition of 1M NaOH.

rapidly solubilized than that in zinc carbonate hydroxide. At 1 h, the ratio of soluble zinc from ZRP was more than 80%, while that from zinc carbonate hydroxide was less than 60%.

Animal experiment. During the throughout feeding period of 4 weeks, no significant difference was observed in the body weight or growth, irrespective of the dietary zinc status; at the end of the experimental period, the mean ± standard deviation of body weight for all the rats (n=18) was 215 ± 12 g. Similarly, the effect of the dietary zinc status was least significant on tissue weight (data not shown).

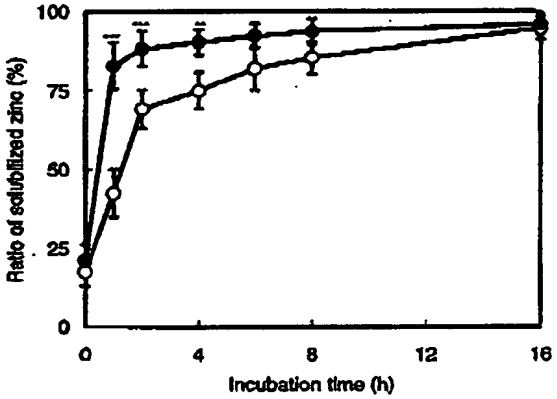

Fig. 1. Effect of the zinc source on zinc solubilization in experimental diets during trypsin digestion

Ten grams of each zinc-supplemented diet were mixed with 50 ml of 0.1N HCl containing 300 mg of a dried powder of porcine pepsin and incubated with shaking at 37℃ for 3 h. After pepsin digestion, the pH of the reaction mixture was adjusted to 7.4 by the dropwise addition of 1M NaOH. Then, 30 mg of crystalline porcine trypsin (5,600 USP trypsin unit/mg, Wako) was added to the mixture and incubated with shaking at 37℃ for a further 16 h. At each time, a portion of the reaction mixture was centrifuged at 6,000 x g for 30 min and the zinc in the supernatant was determined.
Vertical bars denote the SD of the mean for four determinations: those marked with asterisks differ significantly (Student's t-test) from the corresponding control value: ** $p<0.01$, *** $p<0.001$
(○) zinc carbonate hydroxide (●) zinc-rich powder of oyster

The zinc deposition in the plasma, erythrocytes, liver, muscles, and tibia are summarized in Table 4. The zinc deposition in the blood was not increased by dietary zinc supplementation. The liver and muscle zinc tended to increase with the addition of dietary zinc regardless of its source. On the other hand, zinc in the tibia varied according to the zinc source. ZRP supplementation significantly increased the zinc deposition in the tibia, although supplementation with zinc carbonate hydroxide had no effect.

DISCUSSION

Since oysters contain zinc at an especially high level, the utilization of oysters as a component of zinc-rich foodstuffs or a zinc supplement is expected. However, hot water extract of oyster, which is usually used as a nutritional supplement in Japan does not contain zinc level enough for this purpose. The ZRP prepared in this study contained a high concentration of zinc at a level of 7.6%. The powder also contained sodium, phosphorous and calcium at a comparable level to zinc. However, the recommended dietary allowance or intake of these minerals is over 50 times higher that of zinc [1, 7]. Thus, the utilization of ZRP as a zinc supplement does not influence the daily intake of these minerals quantitatively. In addition, the levels of magnesium, iron, copper and manganese were much lower than zinc. Accordingly, ZRP can be used as a nutritional supplement specifically increasing zinc intake.

Many approach have been used to investigate zinc absorption in foods. The *in vitro* approach to zinc absorption has been performed by several researchers [8-10].

Table 4. Zinc deposition in the tissue of rats fed experimental diets

Zinc added	Tissue zinc concentration (μg/g)				
	Plasma	Erythrocytes	Liver	Muscle	Tibia
None	0.29±0.08[a]	2.51±0.11[a]	20.8±1.4[a]	8.09±0.59[a]	13.2±1.6[a]
Zinc carbonate hydroxide	0.29±0.07[a]	2.41±0.10[a]	22.3±0.7[ab]	8.55±0.41[a]	11.8±0.4[a]
Zinc-rich powder of oysters	0.30±0.07[a]	2.55±0.03[a]	24.9±0.7[b]	8.42±0.60[a]	17.3±1.0[b]

Values are the means ± SEM for 6 animals. Means in the same column not sharing a common superscript letter differ significantly ($p<0.05$).

In these reports, solubility and chemical characteristics of zinc in an *in vitro* digest were used for an index of absorption. In the present study, ZRP released zinc more readily than zinc carbonate hydroxide by *in vitro* protease digestion of the zinc-supplemented experimental diets. Accordingly, it is implied that the solubility of zinc in ZRP was higher than zinc carbonate hydroxide in the small intestine.

In the animal experiment, the deposition of zinc from ZRP was significantly higher than that from zinc carbonate hydroxide in the tibia. This result is coincident with that of the *in vitro* digestion test. That is, higher solubility of the ZRP could cause a higher intestinal absorption of zinc from ZRP and resulted in the higher zinc deposition in the tibia of rats fed the ZRP-supplemented diet. Therefore, it can be concluded that the bioavailability of zinc in ZRP is superior to zinc carbonate hydroxide.

Since ZRP is insoluble at a neutral pH, some of the zinc may exist as a form of zinc hydroxide. Nevertheless, a difference between ZRP and zinc carbonate hydroxide was observed in the evaluation of bioavailability. It has been reported that several dietary factors influence zinc absorption [11]. In particular, zinc absorption is enhanced by several dietary ligands and chelators, including low-molecular-weight peptides or amino acids [12-14]. As described in Table 1, the main component of ZRP is acid-soluble Kjeldahl nitrogen, *i.e.* low-molecular-weight peptides and amino acids. These compounds may include ligands or chelators for zinc and promote zinc absorption.

ZRP contained zinc at a particularly high level and its bioavailability was satisfactory. Thus, the use of ZRP as a zinc supplement or for zinc enrichment of food is expected.

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